Evolution, 54(5), 2000, pp. 1687–1698

THE ORIGINS OF PREMATING REPRODUCTIVE ISOLATION: TESTING HYPOTHESES IN THE GRASSHOPPER *CHORTHIPPUS PARALLELUS*

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Abstract.—There are many proposed routes for the origin of premating reproductive isolation, but few systematic studies aimed at testing their relative importance. Accumulated information about the biogeographical history of the European meadow grasshopper, *Chorthippus parallelus,* has allowed us to make a planned series of comparisons among populations aimed at distinguishing the contributions of some of these hypotheses. We have compared the effects on assortative mating of long-term isolation in glacial refugia, founder events during postglacial colonization, and sympatry with a closely related species. A likelihood-based analysis allowed us to separate effects of variation in male and female mating propensity among populations from variation in mate choice leading to assortative mating. All three effects contributed significantly to the overall variation in mating pattern in a set of 21 pairwise comparisons among seven populations. Male cuticular composition, but not other candidate signals, was significantly associated with the level of assortative mating. Of the hypotheses for the origin of reproductive isolation, only the predictions of the founder hypothesis explained a significant amount of the variation in assortative mating. This does not rule out the possiblity that there may be some other explanation. Having established the pattern of divergence, it is possible to generate hypotheses that explain our results at least as well as the founder hypothesis. However, because many such post hoc hypotheses are possible, they cannot be tested with this dataset. On this basis, our results favor the hypothesis that some aspect of the colonization process tends to accelerate divergence in mating signals leading to premating reproductive isolation. This could be accomplished through any one of several mechanisms. Colonization involves many bottlenecks as new populations are established at the edge of the range by long-distance migrants. Genetic effects may be important, but these bottlenecks may also alter the conditions under which mates are found and chosen, as suggested by Kaneshiro. At the same time, the colonizing populations may encounter novel environmental challenges.

Key words.—*Chorthippus parallelus,* colonization, grasshopper, likelihood, mating, Orthoptera, premating isolation, refugia, reproductive isolation, speciation.

Received August 4, 1999. Accepted April 5, 2000.

Speciation depends on the evolution of reproductive isolation between populations of the same species. Many mechanisms for the evolution of such isolation have been proposed (Otte and Endler 1989; Howard and Berlocher 1998), which for animals can be divided into three broad categories. Isolation evolves as an incidental effect of: (1) gradual accumulation of incompatibility by genetic drift in allopatric populations; (2) adaptive divergence in response to environmental variation or sexual selection; and (3) rapid genetic changes associated with founder events (including both extreme drift and changes in the pattern of selection due to population bottlenecks). Divergence initiated by these processes may subsequently be reinforced by selection for assortative mating in zones of contact between divergent populations. Such reinforcement requires partial postmating reproductive isolation before it can operate (Butlin 1998; Noor 1999), but this is not required by the other processes.

The relative importance of these processes can be investigated by comparing patterns of divergence either between or within species (see Tregenza et al. 2000a). Interspecific comparisons may benefit from well-resolved phylogenies (e.g., Coyne and Orr 1989, 1997), but have the inherent drawback that divergence before and after speciation cannot be separated. To identify the causes of speciation, we need to find what the factors are that act before speciation to bring it about, rather than confusing them with processes that may have happened afterward. Comparisons within species have the advantage that observed divergence has occurred prior to speciation and thus may subsequently contribute to the process of speciation itself. Furthermore, assortative mating experiments allow us to quantify reproductive isolation, so we can compare observed patterns of isolation with expectations based on the different theories about the driving forces of speciation.

Past studies have compared patterns of assortative mating with general genetic divergence in a number of species (e.g., salamanders, Tilley et al. 1990; the *Drosophila willistoni* group, Gleason and Ritchie 1998; brown planthoppers, Claridge et al. 1985, 1988). However, there have not been any attempts to simultaneously compare competing hypotheses for the origins of reproductive isolation, presumably because of the requirement for identification of populations of the same species with known and differing evolutionary histories. The accumulated information now available on the biogeographical history of the European meadow grasshopper, *Chorthippus parallelus,* has allowed us to conduct such a study. We have made a planned series of comparisons among populations that enable us to test the extent to which each of the different hypotheses can explain observed patterns of premating reproductive isolation.

Chorthippus parallelus is a flightless, univoltine, gomphocerine grasshopper with a broad distribution throughout Europe. As a result of northward range expansion following the last glaciation (which ended around 10,000 years ago), geographically diverse populations differ considerably in their evolutionary histories. Information about the pattern of range expansion comes from studies of sequence variation in *C. parallelus* from sites all over Europe, using noncoding nuclear DNA (Cooper et al. 1995) and mitochondrial DNA (Lunt et al. 1998). The pattern of sequence similarities be-

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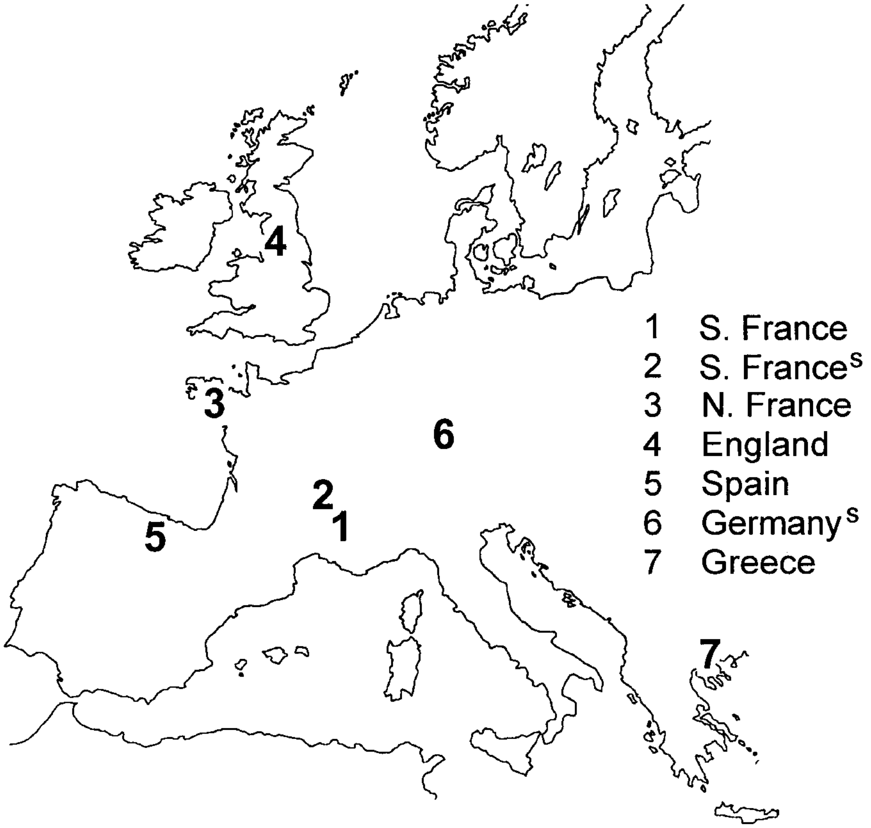

FIG. 1. Collection sites for *Chorthippus parallelus* populations used in our study: (1) southern France 44°21′N, 2°3′E); (2) southern France at a site sympatric with *C. montanus* (45°11′N, 2°13′E); (3) northern France (48°43′N, 3°59′W); (4) England (54°25′N, 3°14′W); (5) Spain (42°50′N, 4°29′W); (6) Germany, also sympatric with *C. montanus* (49°42′N, 10°48′E); and (7) Greece (40°40′N, 23°42′E).

tween areas reveals that, following the last glaciation, Europe was recolonized by the descendants of populations that occupied the Balkan refuge, whereas populations that had been confined to the Iberian Peninsula and Italy did not spread northward out of their refugia (presumably because they were initially prevented from doing so by the Pyrenean and Alpine Mountain ranges). The rate of this range expansion, when compared with observed individual lifetime movement rates within populations (Virdee and Hewitt 1990), suggests that current northern European populations must have undergone repeated founder events. Furthermore, nuclear and mitochondrial sequence diversity is lower in northern Europe than elsewhere, as would be expected if populations had been subject to bottlenecks (Cooper et al. 1995; Lunt et al. 1998).

This variation in evolutionary history between populations allows us to use planned comparisons between populations to compare observed premating reproductive isolation with the patterns predicted by the hypotheses. To quantify premating isolation, we measured assortative mating between seven populations (see Materials and Methods) from Spain, Greece, and central and northern Europe (Fig. 1).

A major difference in evolutionary history between these populations is that the Spanish population is descended from the Spanish refuge, whereas the other populations are all descendants of the Greek refuge. Therefore, if divergence is due mainly to long periods of allopatry, then we would expect to find the greatest isolation between the Spanish- and Greek-descended populations.

Within the descendants of the Greek refuge, some populations have experienced the repeated population bottlenecks associated with range expansion and indicated by the sequence data discussed above. If founder events tend to promote the evolution of reproductive isolation, we would expect differences between descendent populations and refugial populations and possibly differences between the most peripheral populations and those nearer their ancestral refuge.

In terms of investigating whether isolation is promoted by adaptation to differing environments, there is clearly no way to consider all possible sources of selection. However, a potentially powerful source of selection in the local environment is the presence of a closely related species with a similar set of mating signals, which might promote reproductive character displacement (e.g., Howard 1993; Noor 1999). For *C. parallelus,* such a species is the marsh grasshopper, *C. montanus,* which is morphologically almost identical to *C. parallelus* and has similar calling song and cuticular hydrocarbons (Reynolds 1980; Tregenza et. al. 2000a). Two of our central European populations were collected from sites where they are sympatric with *C. montanus* now and are known to have been so for at least 20 years (Voisin 1979; O. von Helversen, pers. comm.), whereas in the other areas, *C. montanus* is absent. We would predict that if sympatry with a

TABLE 1. Results of assortative mating experiments using a male and a female from each of two populations. The χ^2 isolation index (Gilbert and Starmer 1985) takes into account differences in mating propensity. I is the parameter in model 16 that measures the tendency of a female to mate with the male of its own population in preference to the other male in the mating trial. It has been multiplied by four so that it is on the same scale (-1 to $+1$) as the χ^2 isolation index. Positive indices indicate assortative mating. A previous preliminary analysis of our assortative mating experiments using matrix correlations, in which χ^2 isolation indices were presented, contained errors (Butlin and Tregenza 1998). These errors do not change the significance of any of the Mantel correlations between pattern of mating and phenotypic or genetic divergence calculated in that analysis.

		Mating type						
Population 1	Population 2	Male 1 × female 1	Male 2 × female 2	Male 2 × female 1	Male 1 × female 2	χ^2 isolation index	I (× 4)	Genetic distance[1]
S. France	S. France^S	4	10	5	11	−0.067	−0.115	0.000
S. France	N. France	5	5	4	11	−0.122	−0.198	0.029
S. France	England	7	8	5	5	0.198	0.196	0.041
S. France	Spain	10	7	10	10	−0.088	−0.019	0.217
S. France	Germany	4	9	7	4	0.056	0.030	0.017
S. France	Greece	10	10	4	5	0.380	0.392	0.090
S. France^S	N. France	8	10	2	4	0.500*	0.511	0.029
S. France^S	England	4	6	11	3	−0.063	−0.108	0.041
S. France^S	Spain	9	8	2	3	0.545*	0.556	0.217
S. France^S	Germany	8	5	5	5	0.113	0.074	0.017
S. France^S	Greece	14	3	0	4	0.381*	0.439	0.090
N. France	England	8	12	2	5	0.472*	0.382	0.073
N. France	Spain	3	5	8	9	−0.365	−0.356	0.165
N. France	Germany	11	12	10	6	0.189	0.204	0.013
N. France	Greece	13	8	4	2	0.527*	0.516	0.068
England	Spain	6	11	5	4	0.272	0.347	0.148
England	Germany	7	5	2	6	0.230	0.231	0.017
England	Greece	16	3	1	4	0.306*	0.493	0.036
Spain	Germany	6	6	5	6	0.045	0.086	0.189
Spain	Greece	17	7	4	1	0.547*	0.541	0.131
Greece	Germany	12	6	3	2	0.499*	0.485	0.049

[1] From Cooper et al. (1995).

* Individual χ^2 isolation index is significantly different from random mating at $P < 0.05$.

close relative tends to drive the evolution of premating isolation between conspecific populations, the *C. parallelus* populations sympatric with *C. montanus* would mate assortatively when placed with members of other populations. Such a scenario has been proposed to explain premating isolation between populations of *Drosophila mojavensis* sympatric with *D. arizonae* and allopatric populations (Zouros and d'Entremont 1980).

In addition to examining which aspects of evolutionary history are associated with reproductive isolation, we also looked at whether patterns of trait divergence can explain variation in premating reproductive isolation. Data from our previous study of male and female morphology, cuticular composition (a putative contact pheromone; Butlin 1998), and male calling song, from the same seven populations, were incorporated into a model of the pattern of assortative mating (see Materials and Methods). This allowed us to examine which aspects of the mating system are likely to contribute to isolation.

MATERIALS AND METHODS

We collected a minimum of 40 mated adult female grasshoppers from each of seven sites across Europe (Fig. 1). We allowed the females to lay eggs and subsequently reared the offspring under standard conditions in the laboratory, as described by Tregenza et al. (2000a). On the day each grasshopper became an adult, it was transferred from the 30-cm high, 24-cm diameter larval rearing cage into a smaller cage (22-cm high, 9-cm diameter) that contained a maximum of four other individuals of the same sex and population and a pot of *Dactylis glomerata* grass, which was replaced every two to three days. Each newly eclosed adult was given a unique mark using a combination of dots of different colored acrylic paints that were placed on either the right or left side in the front or back half of the pronotum

Assortative Mating Experiments

Two male-female pairs from two different populations were placed in a 40 × 30 × 20cm arena, which was maintained at a minimum of 25°C by an overhead 60-W lamp. The base of the arena was covered in fresh grass and the sides with foam to minimize reflection of male calls. Individuals of the same sex were matched for age to the same day when four days old and as closely as possible if older (mean age difference between members of the same sex was 6% of mean age). Only virgin females were used. The grasshoppers were observed for a minimum of 3 h or until a mating occurred, at which point the experiment was stopped. The 1:1:1:1 design of the experiments means that mating propensity can be considered in the analysis, but still allows individuals to choose among potential mates, rather than only being able to choose whether to mate, as in 1:1 experiments. Members of the same sex were not observed to interact physically with one another, although males could have influenced each other through calling songs. A total of 547 assortative mating experiments were carried out with a minimum of 20 crosses between each pairwise combination of populations (Table 1;

TABLE 2. Maximum-likelihood parameter estimates from model 14 (support limits).

Population	Relative male mating propensity (v_1–v_7)	Relative female mating propensity (w_1–w_7)	Position on isolation axis (m_1–m_7)
S. France (reference)	1.0	1.0	0.0
S. France^{S}	1.138 (0.846–1.390)	1.714 (1.311–2.020)	0.553 (0.283–0.803)
N. France	0.824 (0.568–1.021)	1.400 (1.183–1.695)	−0.301 (−0.522–−0.104)
England	1.241 (1.032–1.549)	1.176 (0.887–1.442)	0.664 (0.375–0.836)
Spain	0.961 (0.758–1.202)	1.254 (0.984–1.559)	−0.235 (−0.445–−0.014)
Germany^{S}	0.952 (0.719–1.158)	1.183 (0.897–1.371)	0.125 (−0.140–0.358)
Greece	0.365 (0.200–0.545)	0.500 (0.322–0.696)	1.091 (0.916–1.336)

not a minimum of 25, as erroneously stated in Butlin and Tregenza 1998).

Data Analysis

The data consist of all pairwise combinations of the seven populations. Therefore, although there are only seven populations, there are 21 independent tests of mating pattern, reflecting the fact that reproductive isolation is not a feature of populations, but the result of interactions between populations. For each test, there is some total number of trials (20–39, mean = 26.0) each of which has four possible outcomes. Results are summarized as counts, n_{ij}, of the trials ending in mating between a male from population i and a female from population j. Deviations from random mating may be caused by mate choice or by differences between populations in male mating propensity or female mating propensity. A preliminary analysis of these data (Butlin and Tregenza 1998) using matrix correlations did not allow us to separate these effects, because, in a single test, there are insufficient degrees of freedom. However, Davies et al. (1997) have introduced a likelihood-based method that uses data from several tests involving the same populations to distinguish between male choice and mating propensity. This approach is ideal for the present dataset because males in the 21 tests come from only seven populations and so variation in male mating propensity can be modeled with just six parameters (with one population used as a reference with propensity set to one). The same is true for variation in female mating propensity. Even if a very complex model of mate choice is employed, that is, a separate level of assortative mating for each of the population pairs, only 21 additional parameters are required and 51 degrees of freedom are available ([21 × 3] − 6 − 6 = 51). Less complex models of mate choice can also be fitted, starting from a single level of assortment that applies to all population pairs (i.e., all populations have the same tendency to mate assortatively regardless of the other population they are paired with). Each increase in complexity can be tested for an improvement in fit because twice the increase in log-likelihood asymptotically follows the χ^2 distribution with degrees of freedom equal to the number of additional parameters (Edwards 1972). When an additional parameter was added to an existing model to create a new model, all parameters were reoptimized.

TABLE 3. Maximum-likelihood estimates of isolation parameters (see Fig. 2 for significance levels and Materials and Methods: Data Analysis for parameter definitions).

Model	Isolation estimate (× 4)
Uniform isolation (2)	$I = 0.236$
Uniform isolation with male and female mating propensity (8)	$I = 0.221$
Refuge (9)	$I_{\mathrm{SpB}} = 0.175$, $I_{\mathrm{BB}} = 0.245$
Founder (10);	$I_{\mathrm{SpB}} = 0.147$, $I_{\mathrm{BF}} = 0.438$, $I_{\mathrm{FF}} = 0.135$
Founder (long 11)	$I_{\mathrm{SpB}} = 0.154$, $I_{\mathrm{BS}} = 0.428$, $I_{\mathrm{BL}} = 0.485$, $I_{\mathrm{LL}} = 0.398$, $I_{\mathrm{LS}} = 0.138$, $I_{\mathrm{SS}} = 0.057$
Sympatry (12)	$I_{\mathrm{SpB}} = 0.154$, $I_{\mathrm{SySy}} = 0.136$, $I_{\mathrm{ASy}} = 0.208$, $I_{\mathrm{AA}} = 0.289$
Linear isolation (14)	See Table 2
Independent symmetrical isolation (16)	See Table 1

Our populations were chosen on the basis of information about their biogeographical history to test competing hypotheses about the origins of premating reproductive isolation (see introduction). These hypotheses can be expressed as models for the pattern of mate choice across the 21 tests. In addition, estimates are available for the pairwise distances between populations for several sets of characters that might influence mate choice or correlate with the level of premating isolation, namely male song, male cuticular composition, female cuticular composition, male and female morphology (Tregenza et al. 2000a), and a noncoding nuclear genetic marker (Cooper et al. 1995). The relationship of each of these distances to levels of assortative mating can be incorporated into the models.

The following sequence of models has been tested:

Model 1.—Null model, that is, equal probability (p) of the four outcomes $p_{ii} = p_{ij} = p_{ji} = p_{jj} = 0.25$.

Model 2.—Uniform isolation in all pairwise combinations of populations with no variation in male or female mating propensity among populations. This model requires one parameter, I, such that: $p_{ii} = 0.25 + I$, $p_{ij} = 0.25 - I$, $p_{ji} = 0.25 - I$, $p_{jj} = 0.25 + I$.

This is a model of symmetrical assortative mating: asymmetry is considered below (model 15). I ranges from $+0.25$ for complete assortment to -0.25 for completely disassortative mating (as in Davies et al. 1997). Multiplying I by four gives a value comparable to the commonly used isolation indices (Gilbert and Starmer 1985)

Model 3.—Male mating propensity (v) varies among populations. The mating propensity of population 1 (southern France) was set to 1.0 and the propensities of the other populations, $v_2 \ldots v_7$ (six parameters), were fitted as in Davies et al. (1997), that is: $p_{ii} = v_i/(v_i + v_j)$, $p_{ij} = v_i/(v_i + v_j)$, $p_{ji} = v_j/(v_i + v_j)$, $p_{jj} = v_j/(v_i + v_j)$.

Model 4.—Female mating propensity (w) varies among populations. This is equivalent to male mating propensity, but with $w_1 = 1.0$ and $w_2 \ldots w_7$ fitted such that: $p_{ii} = w_i/(w_i + w_j)$, $p_{ij} = w_j/(w_i + w_j)$, $p_{ji} = w_i/(w_i + w_j)$, $p_{jj} = w_j/(w_i + w_j)$.

Model 5.—Male mating propensity plus uniform isolation combining models 2 and 3 (seven parameters), for example: $p_{ii} = [v_i/(v_i + v_j)] + I$,

Model 6.—Female mating propensity plus uniform isolation combining models 2 and 4 (seven parameters), for example: $p_{ii} = [w_i/(w_i + w_j)] + I$,

Model 7.—Male and female mating propensity combining models 3 and 4 (12 parameters), for example: $p_{ii} = [v_i/(v_i + v_j)][w_i/(w_i + w_j)]$

Model 8.—Male and female mating propensity plus uniform isolation combining models 2 and 7 (13 parameters) to give expectations of the form, for example: $p_{ii} = [v_i/(v_i + v_j)][w_i/(w_i + w_j)] + I$ and so on.

All subsequent models incorporate variation in male and female mating propensity (but new estimates of the parameters, v_i and w_i, are made in each case).

Model 9.—Refuge: If isolation is primarily accumulated due to long-term divergence in refugia, strong isolation is expected between the Spanish population and the other populations, whereas weak isolation is expected among populations derived from the Balkan refuge (see introduction). The parameter I in model 8 is replaced by two parameters, I_{SpB} (average isolation between the Spanish population populations derived from the Balkan refuge) and I_{BB} (average isolation among populations derived from or in the Balkan refuge); (14 parameters in total).

Model 10.—Founder: If isolation is accumulated during colonization as a result of population bottlenecks, then isolation among populations derived from the Balkan refuge may differ from isolation between these populations and the Greek population. The parameter I_{BB} in model 9 is replaced by two parameters, I_{BF} (average isolation between the Greek population and populations derived from the Balkan refuge) and I_{FF} (average isolation among derived populations); (15 parameters). The parameter I_{SpB} is unchanged because it is not clear whether founder populations diverging from their ancestral populations will also tend to diverge from unrelated populations. For instance, if mates are recognized by the ratio of two cuticular components, then a change in the concentration of these chemicals may lead to divergence from one population, but convergence with another.

Model 11.—Founder (long): Following from the argument of model 10, populations with long colonization routes (England and northern France) may be more isolated from one another, and from those with short colonization routes, than the short-route populations are among themselves. The parameter I_{FF} in model 10 is replaced by three parameters, I_{LL}, I_{LS} and I_{SS}, and parameter I_{BF} is replaced by two parameters, I_{BS} and I_{BL}:(isolation between populations with long colonization routes: England and northern France); I_{LS} (average isolation between these populations and others derived from the Balkan refuge), and I_{SS} (average isolation between populations with short colonization routes from the Balkan refuge); I_{BF} by I_{BS} (average isolation between the Greek population and derived populations with short colonization routes), and I_{BL} (average isolation between the Greek population and derived populations with long colonization routes); (18 parameters).

Model 12.—Sympatry with *C. montanus* may result in character displacement and thus increased isolation between sympatric and allopatric populations and/or among sympatric populations. The parameter I_{BB} in model 9 is replaced by three parameters, I_{SySy} (isolation between sympatric populations: southern France$^{\mathrm{S}}$ and Germany$^{\mathrm{S}}$), $\mathrm{I}_{\mathrm{ASy}}$ (average isolation between sympatric populations and other populations derived from the Balkan refuge), and I_{AA} (average isolation among allopatric populations derived from the Balkan refuge); (16 parameters).

Model 13.—Trait divergence among populations in other characters may explain variation in levels of isolation between pairs of populations. If so, the greater the distance between populations, the stronger will be the assortative mating. Arnold et al. (1996) argued that, as two populations diverge in a signal character and associated preference, the probability of mating between individuals from different populations will decline exponentially. Therefore, the effect of divergence was modeled by reducing the expected frequencies of interpopulation matings to: $p_{ij} = [v_i/(v_i + v_j)][w_j/(w_i + w_j)]\exp(-a.d_{ij}^2/2)$ and $p_{ji} = [v_j/(v_i + v_j)][w_i/(w_i + w_j)]\exp(-a.d_{ij}^2/2)$ and adjusting p_{ii} and p_{jj} such that (Σp remained equal to one. The parameter a is a scaling parameter and d_{ij} is the measured distance between populations i and j for the character in question. Each model of this type has 13 parameters because I is replaced by a. These models represent alternatives to model 8, having the same number of parameters, rather than being more complex members of a nested series. The effects are considered significant if there is an improvement of more than two log-likelihood units in comparison with model 8.

The following distances were examined: 13a, *male cuticular composition*; 13b, *female cuticular composition*; 13c, *male song*; 13d, *male morphology*; and 13e, *female morphology*, all using Mahalanobis distance matrices derived from the data in Tregenza et al. (2000a).

13f, *genetic distance* uses K_{ST} distances from Cooper et al. (1995). Our sample sites are not identical to those of Cooper et al., but they are often close and frequently surrounded by their more numerous sites. They combined groups of sites into regions before calculating K_{ST}-values and we have used these between-region distances.

Model 14.—Rather than fitting a set of distances for some character that might influence isolation, it is possible to consider a model in which populations are placed on a linear axis such that the isolation between any pair of populations is predicted by their separation on this axis alone. This is equivalent to model 13, except that the probability of an interpopulation mating is reduced by the factor $\exp[(m_i - m_j)^2/2]$. Population 1 was used as a reference point with $m_1 = 0.0$ and positions of the other populations, $m_2 \ldots m_7$, were allowed to vary. This model has 18 parameters. In comparison with the model of Arnold et al. (1996), it assumes that female preference mean exactly matches the male trait mean in each population and both trait and preference have variances equal to one. It models symmetrical assortative mating.

Linear isolation implies that the level of isolation between a pair of populations, AC, is predictable from the isolation between pairs AB and BC. This need not be the case. For example, isolation between A and B may be due to divergence in song, whereas isolation between B and C may be due to divergence in male cuticular composition. Isolation between A and C then depends on the way in which these two signal channels interact. Also, the linear isolation model will only fit well if signal and preference variation is closely correlated among populations.

Model 15.—Linear male and female separation provides for the possibility that mean female preference does not match the mean of the male trait distribution within each population. This is one way in which asymmetrical isolation might be generated (Arnold et al. 1996). Instead of a single value for each population on the isolation axis, there are separate values for males and females. This means that males of population i may be closer to females of population j than j males are to i females, resulting in an asymmetric pattern of matings. Using $m_1 = 0.0$ as a reference point again, there are now 13 parameters for mate choice, $m_2 \ldots m_7$ and $f_1 \ldots f_7$, giving 25 parameters in total. Interpopulation matings are reduced by the factor $\exp[(m_i - f_j)^2/2]$ and intrapopulation matings by $\exp[(-m_i - f_i)^2/2]$, and rescaled to give a total probability of one.

Model 16.—*Independent symmetrical isolation* for each of the 21 pairwise comparisons, with variation in male and female mating propensities (33 parameters).

Model 17.—*Complete model:* Whether significant unexplained variation remains after fitting any of these models can be determined by comparing the log-likelihood with the log-likelihood of a complete, 63-parameter model in which observed and expected values are equal.

Some of these models require the simultaneous estimation of many parameters that potentially interact in their effects on the likelihood. Therefore, models were fitted using an implementation of the Metropolis algorithm (Kirkpatrick et al. 1983), written in Genstat (Lane et al. 1995), to search for maximum-likelihood parameter values. In this method, random changes in parameter values are made iteratively. Changes are accepted if they increase the likelihood. If they decrease the likelihood, they are accepted with a probability that depends on the change in likelihood and a ''temperature'' parameter. Initially, at high temperatures, the probability of acceptance is high, allowing the process to explore a wide range of possible parameter combinations. As the process proceeds, the temperature is reduced and parameter estimates move more consistently toward the region of maximum likelihood. The algorithm provides an effective means of dealing with simultaneous estimation of many parameters. The procedure was checked for models with six or fewer parameters using the optimization algorithm provided in Genstat (FITNONLINEAR). For more complex models, fits were checked by rerunning the Metropolis algorithm from different starting points. Support limits for parameter estimates (the values at which the log-likelihood is two units below the maximum, approximately equivalent to 95% confidence limits; Edwards 1972) were obtained by calculating the log-likelihood for large numbers of parameter combinations (more than 2500) in the region of the maximum-likelihood values. The Metropolis program was modified so that, starting from the maximum-likelihood parameter combinations, all random parameter changes were accepted until the likelihood fell to more than two units below the maximum. A new run was then initiated at the maximum-likelihood combination.

RESULTS

Assortative Mating Experiments

The full results of our assortative mating experiments are given in Table 1.

Data Analysis

There was significant variation among populations in both male and female mating propensities, regardless of whether they were included in models alone, together, with mate choice, or without it (Fig. 2, models 1–8). Estimates for male and female mating propensities from model 14 (Fig. 3, Table 2) are positively correlated ($r = 0.73$, $0.10 > P > 0.05$). Thus, some populations have generally lower mating activity under laboratory conditions than others. The strongest effect is the low mating activity of both sexes from the Greek population.

There was also significant mate choice (Fig. 2, compare models 1 and 2 or 7 and 8) and this varied among population pairs (compare models 8 and 16, see Table 1 for estimates of I in model 16). A substantial proportion of the variation in levels of assortative mating among pairs of populations was explained by the linear isolation axis (model 14, see Table 2 for estimates of m_1–m_7), suggesting that mate choice is based on a single trait or a set of traits that vary together among populations. The most extreme population on the isolation axis was the Greek population, whereas the populations from southern France, northern France, Bavaria, and Spain clustered together at the opposite end of the axis (Fig. 4). Allowing separate positioning of males and females on the isolation axis (model 15) did not improve the fit, suggesting that male and female components of mate recognition correspond within populations and that any asymmetrical isolation is not generated by the mechanism proposed by Arnold et al. (1996)

The hypothesis that premating isolation is primarily due to long-term divergence in refugia is not supported by the data (Fig. 2, compare models 8 and 9) nor is there evidence for an effect of sympatry with a close relative (model 12). However, the founder model (10) does provide a significant improvement, reflecting the extreme position of the Greek population on the isolation axis. Populations derived from the Greek refugium through postglacial range expansion are

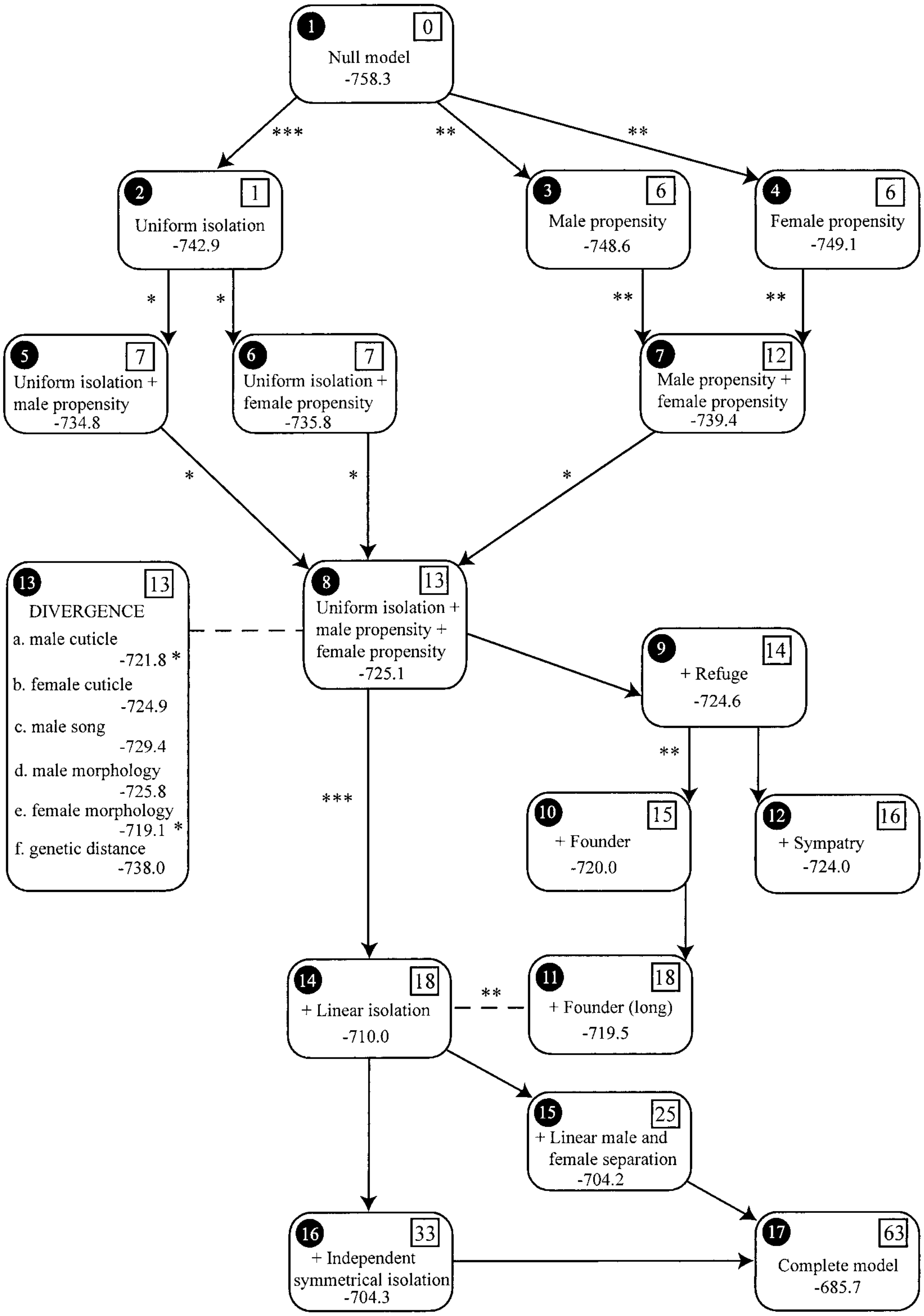

FIG. 2. Summary of the model-fitting process. Each model is represented by a box. The number in the top left corner is the model number (see Methods: Data Analysis). The number in the top right corner is the number of parameters fitted, and the log-likelihood is given at the bottom. Solid arrows connect nested models (a simpler model leading to a more complex model), dashed lines connect alternative models (with different parameters rather than an increase in complexity). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

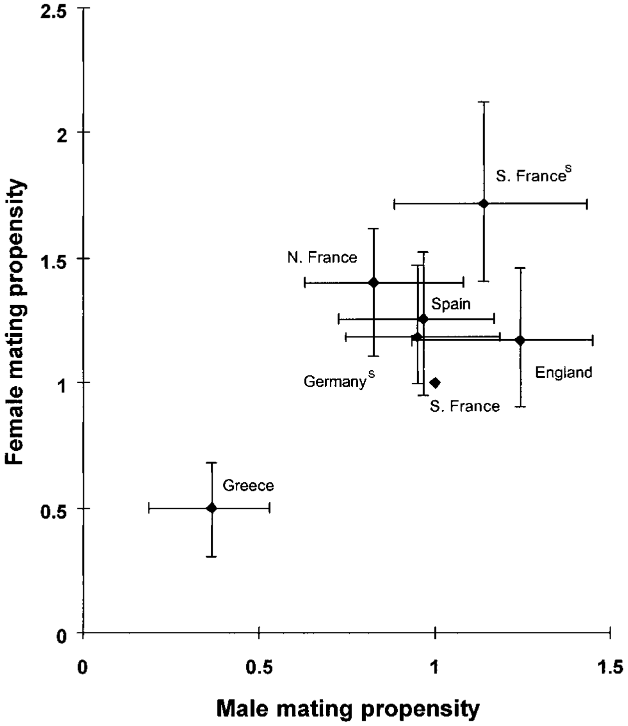

FIG. 3. Male and female mating propensity estimates from model 14, with support limits. Southern France was used as the reference population, with both male and female mating propensity set to one.

weakly isolated from one another: on average ($I_{FF} \times 4 = 0.035$), but strongly isolated from the population that remains in the refugial area ($I_{BF} \times 4 = 0.438$). Adding the effect of long-distance colonization (model 11) does not provide a significant improvement, although I_{LL} is greater than I_{SS}, as expected, because the UK population is more isolated than any other population apart from Greece.

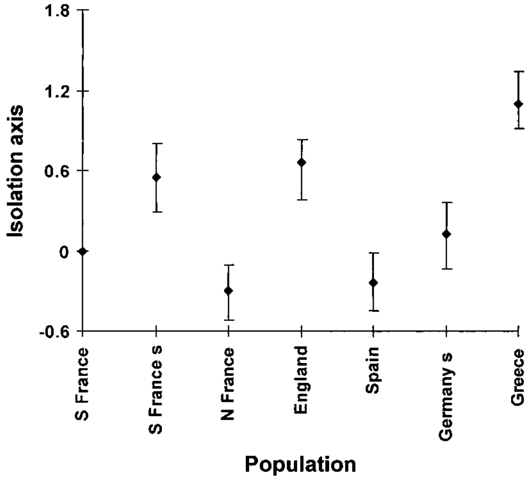

FIG. 4. Estimates of population positions on the linear isolation axis from model 14, with support limits. The strength of assortative mating between a pair of populations is proportional to the distance apart of the populations on the isolation axis. Southern France was used as the reference population, with a value of zero on the isolation axis.

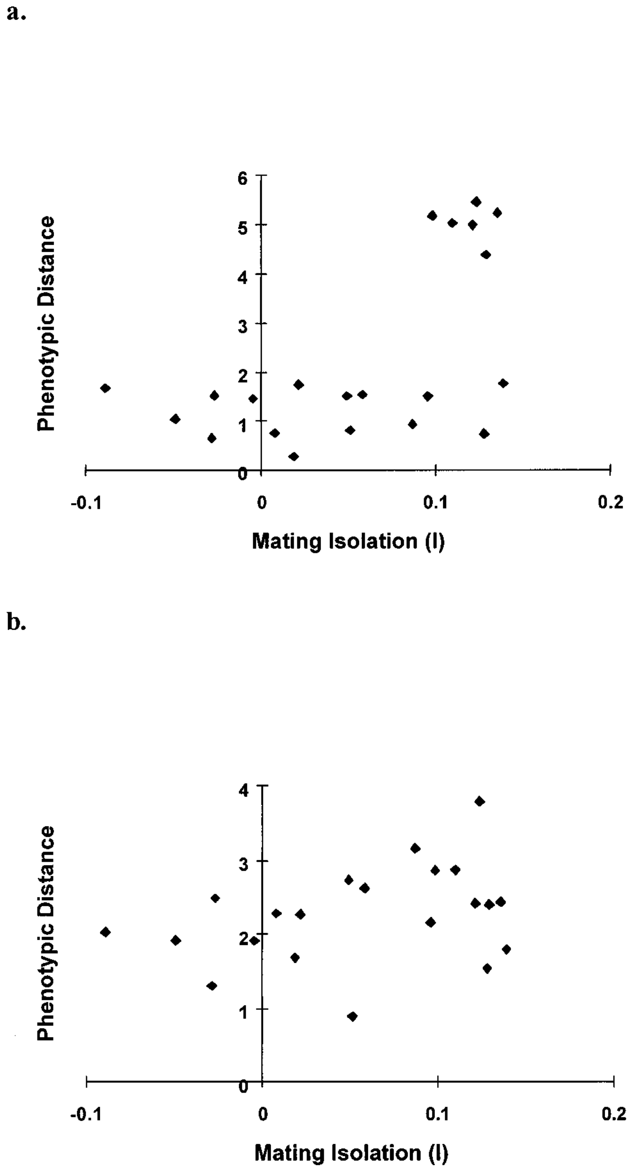

FIG. 5. The relationship between assortative mating, the isolation parameter *I* for each pair of populations as estimated from model 16, and (a) divergence in female morphology or (b) divergence in male cuticular hydrocarbon blend. Divergence is expressed as the Mahalanobis distance between each pair of populations. In (a), all comparisons with distance > 4 involve the population from Greece. In (b), isolation is not dominated by a single population, although if the 21 crosses are ranked according to distance, the Greek population has the first, third, fourth, eighth, ninth, and 10th highest distances.

Only two of the six trait divergence models produced better fits than the uniform isolation model: male cuticular composition (13a) and female morphology (13e). The apparent effect of female morphology is largely accounted for by the Greek population (Fig. 5a) and is likely to be due to the larger body size of individuals from this stock (Tregenza et al. 2000a). Males from this population are also larger than those from other localities, but size contributes less to the overall morphological distance among males than among females. In an analysis of individual mating trials (T. Tregenza and R. K. Butlin, unpubl. ms.), neither the absolute nor the relative sizes of male and female grasshoppers had a detectable influence on the outcomes. Therefore, it seems likely that the association observed at the population level is not causative. However, there is evidence that cuticular compounds function as contact pheromones (Ritchie 1990; Buckley 1998; Butlin 1998), they are sexually dimorphic, and vary considerably among populations (Tregenza et al. 2000b). The effect of male cuticular composition on isolation is not dominated by a single population, although the Greek population does make a larger contribution than the other populations (Fig. 5b).

Song, which is normally considered a major component of the mate recognition system in gomphocerine grasshoppers, varies little among these populations relative to within-population variation (Tregenza et al. 2000a) and so would be unlikely to explain the pattern of assortment. It is striking how poor a fit is provided by genetic distances. This reflects the poor fit of the Refuge hypothesis (model 9), because the Spanish population is the most divergent genetically.

Isolation indices from model 16 closely parallel the χ^2 index of isolation (Gilbert and Starmer 1985; mean $I \times 4 = 0.217$, mean χ^2 index $= 0.223$, $r = 0.977$). This supports Gilbert and Starmer's conclusion that the χ^2 index is robust to substantial variation in mating propensity among populations, such as we see here. However, the parallel variation in male and female propensity among grasshopper populations may be less of a problem than independent or negatively correlated variation of the two sexes, which may generate the appearance of asymmetrical isolation. The complete (63-parameter) model does not produce a significant improvement on either the linear isolation model (model 14; $-2\Delta\text{LL} = 48.6$, df $= 45$) or the 33-parameter independent symmetrical isolation model (model 16; $-2\Delta\text{LL} = 37.2$, df $= 30$), indicating that the simpler models explain most of the variation in mating pattern.

Discussion

A difficulty in examining patterns of reproductive isolation between populations is that it is possible to come up with plausible explanations for almost any pattern and difficult to compare observed patterns with a null hypothesis. Therefore, our aim has been to derive predictions a priori based on the major hypotheses that have been suggested to explain the evolution of reproductive isolation and to examine which of these hypotheses best explains our data. Of course it is possible that none of the available hypotheses is the true explaination for the pattern of divergence we observe. Because there are an unlimited number of post hoc hypotheses, we cannot formally test whether any of them provides a better explaination, although this possibility should be borne in mind.

It is clear from Table 1 that although no cross shows complete premating isolation, there is assortative mating between a number of populations. Furthermore, there is considerable variation between populations in mating propensity. In crosses using the Greek population, there are fewer than half as many homogametic matings between Greek individuals as between grasshoppers from other populations. Why such differences in mating propensity exist is not clear. One possibility is that mating propensity cannot be treated as completely independent of assortative mating. The Greek population may appear to have the lowest mating propensity because there is some general stimulatory effect of conspecifics, providing they are sufficiently similar in mating signals, which increases the likelihood of both homo- and heterospecific matings. This could be examined by conducting experiments using only members of a single population. Such an effect might tend to increase reproductive isolation in already isolated populations, because outcrossing will be opposed by both discrimination against divergent individuals and by reduced likelihood of mating due to lack of stimulation.

The higher mating propensity of descendant populations when compared to the refugial Greek population is in line with Kaneshiro's (1989) prediction that populations subject to founder effects will show increased mating propensities (because of increased risk of failing to mate at all). However, our results do not generally provide any evidence for the Kaneshiro effect because the Spanish refugial population does not have a lower mating propensity than descendant populations and the more peripheral populations do not differ from those nearer the Greek refuge. This may be because the populations have passed through the transient period of low population density during which mate choice is relaxed.

The finding that a substantial proportion of the variation in levels of assortative mating is explained by a model in which populations are arranged on a linear axis with the distance between pairs indicating their isolation suggests that a single trait or set of covarying traits is responsible for isolation. Of the mating system components we measured, only male cuticular pheromones are plausible candidates for such a character. However, there remains a large difference in the explanatory power of the model based on divergence in cuticular composition and that in which populations are arranged on a linear axis. Because we do not know which aspects of cuticular composition are used in signaling, it is likely that our description of the male cuticle combines several aspects that may be under differing selection pressures (Tregenza et al. 2000b). Our measure of cuticular composition may obscure the degree of divergence in cuticular pheromones used as mating signals. Additional work is needed to identify which aspects of cuticular composition are used as mate signals and to examine covariation between these specific aspects and assortative mating.

The finding that no improvement in fit is gained from allowing separate positioning of males and females (model 15), suggests that isolation is not strongly asymmetrical between the sexes. This contrasts with the prediction of Arnold et al.'s (1996) model, which predicts that there will be sexual asymmetry, particularly in the intermediate stages of divergence (comparable with the divergence between populations in our study). Similarly, Kaneshiro's (1989) prediction that founder events will tend to create asymmetries in sexual isolation is not supported by our data.

The only hypothesis for an aspect of evolutionary history which contributes significantly to our premating isolation model, is the founder hypothesis (model 10). The explanatory power of the founder hypothesis rests largely on the isolation of the Greek population from its descendants in northern Europe. However, assortative mating between the Greek population and northern European populations is not solely responsible for the fit of the founder model. For instance, the peripheral northern France population mates assortatively with two of the other Balkan descendent populations as well as with the Greek population. If this population were omitted, then we might not be able to exclude other hypotheses for the origins of reproductive isolation. The finding that there is strong premating isolation between populations recently descended from the same refuge is in itself strong evidence that long periods of allopatry are not necessary for the evolution of reproductive isolation and is an observation independent of the Greek sample.

Although the founder hypothesis clearly explains our data better than any of the other hypotheses tested, the fact that this is mainly due to the isolation of the Greek population gives grounds for caution. Simple founder scenarios might predict similar reproductive isolation among founder populations as between founder populations and their ancestral populations. Also, one might expect equally great isolation between the Spanish population and the founder populations, as between the Greek population and the founder populations. There is some isolation between the Spanish population and the Balkan descendant populations (for instance between populations 2 and 5, Table 1). The lack of improved fit of the model 9 (the refugial model) over model 8 (uniform isolation between populations) reflects the fact that there is not significantly more isolation between the Spanish population and all the other populations than there is among all the populations (model 8). In other words, there may be isolation between the founder populations and Spain, but it is not enough to improve the fit of the model significantly beyond model 9. However, when differences between the Greek population and its descendants are allowed for (as in model 10), there is a significant improvement of the fit to the model. It is possible that the lower isolation between Spain and northern Europe reflects limited degrees of freedom for divergence in mate preferences and traits so that increased isolation from one population may result in decreased isolation from another. This may also explain the lower isolation among Balkan descendant populations than between these populations and Greece. The possibility is supported by our finding that the complete model does not provide a significantly better fit to the data than the linear isolation model.

The influence of the strongly isolated Greek population invites alternative hypotheses that might explain our data. For instance, there might be something unique about the environment of the Greek population that has driven its divergence. The fact that we cannot formally test this hypothesis reflects a fundamental difficulty in this type of study. One would need a much larger number of replicate populations to reduce the influence of each individual population.

Our analysis suggests that, of the hypotheses we tested, the only influential effect is that some aspect of the colonization process tends to accelerate divergence in mating signals leading to premating reproductive isolation. Colonization involves numerous bottlenecks as new populations are established at the edge of the range by long-distance migrants. Genetic effects of these bottlenecks may be important, but at the same time, colonizing populations may encounter novel environmental challenges. We examined one such environmental effect, the presence or otherwise of a closely related species, *C. montanus,* and found no difference in mate choice (or mating signals; Tregenza et al. 2000a). However, we cannot rule out other environmental effects because it is not possible to examine every potential environmental selection pressure.

An assumption of our approach is that individual populations are representative of other populations in their broad geographical area. This raises the question of whether the Greek population is typical of the Balkan refugial area. Cooper et al. (1995) examined samples from nine sites in the putative Balkan refugial area in their genetic analysis. They found some variation between three regions (northern and southern ''mainland'' Greece and Peleponnese Greece), but this was less than the variation among northern European populations derived from the refuge and much less than the variation between refugia (Spain, Italy, Balkans, Turkey, and Russia). Therefore, it is unlikely that the Balkan refuge harbors highly divergent populations that have contributed differentially to the colonization of northern Europe.

A previous study examining patterns of divergence between populations in potential mating signals and in morphology (Tregenza et al. 2000a) also found evidence for differences between populations derived from the same refuge. However, unlike the pattern of premating isolation described here, a significant proportion of variance in potential signal traits was also explained by separating the descendants of different refugia. Similarly, genetic sequence variation also reveals differences between Spanish populations and those derived from the Greek refuge (Cooper et al. 1995; Lunt et al. 1998). Our assortative mating results indicate that neither genetic divergence nor trait divergence provide reliable markers for premating reproductive isolation (models 13a–f). Few previous studies of the relationship between premating isolation and genetic or phenotypic divergence exist, but those that do suggest that our species it not atypical. In salamanders (Tilley et al. 1990), there is a good match between genetic divergence and assortative mating, but other taxa, such as *Drosophila* (Coyne and Orr 1989, 1997), tend to show a weaker relationship and many show no relationship at all. Gleason and Ritchie's (1998) study within the *D. willistoni* group and studies of the brown planthopper (*Nilaparvata lugens*; see Butlin and Tregenza 1998) both found that divergence in premating isolation was not correlated with general genetic and phenotypic divergence between populations or species pairs. Our study also finds premating isolation between genetically similar groups but also reveals a lack of assortative mating between genetically and phenotypically distinct populations. In this respect, our results are similar to the pattern found in three closely related species of butterfly fish (Chaetodontidae; McMillan et al. 1999), two of which show substantial color pattern differences, but no assortative mating. The observed lack of correlation between the pattern of variation in phenotype and genetic sequence and the pattern of variation in mating preference rests partly on the lack of assortative mating between our Spanish population and most of the populations in the rest of Europe. This contrasts with previous work (Ritchie et al. 1989; Butlin and Ritchie 1991), which found assortative mating in populations of *C. parallelus* from either side of the Pyrenean Mountains that separate France and Spain. The explanation for this result almost certainly lies in the different Spanish and French populations used in our study compared to previous studies. It is worth noting that a simple χ^2 measure of isolation between one of our nonrefugial populations, the southern France^s population, indicates significant assortative mating with our Spanish population, whereas the other one does not (Table 1). Therefore the differences between our results and previous work may be due to divergence among French populations, resulting from some aspect of the colonization process. Isolation observed in some comparisons across the Pyrenees may be due to the long colonization route of the French populations rather than because of differences between the French and Spanish populations resulting from their being descendants of different refugia.

Additional evidence that there are significant differences between populations descended from the Balkan refuge comes from comparison of our results and those of Dagley et al. (1994). In their study, Dagley et al. found evidence for assortative mating in all crosses between populations of *C. parallelus* from Mull (a Scottish Island), Jersey, in the English Channel, and Aunat (20 km north of the Pyrenees in southern France). This contrasts with the lack of assortative mating between our United Kingdom population and populations in southern France.

Our study is the first systematic attempt to compare competing hypotheses for the origins of premating reproductive isolation. Our results show that long periods of allopatry are not necessary for the evolution of substantial reproductive isolation and that other factors may be more important. Our analysis suggests that processes associated with range expansion may be particularly important in creating reproductive isolation, but there remain substantial levels of unexplained isolation between populations with similar evolutionary histories, suggesting that the evolution of reproductive isolation can occur rapidly. Similar studies of other species would help to confirm the generality of our findings and to begin to build up a picture of the relative importance of different evolutionary processes driving reproductive isolation and speciation.

ACKNOWLEDGMENTS

We thank the following people for invaluable assistance in collecting and/or rearing grasshoppers: J. Bridle, S. Buckley, N. Flanagan, K.-G. Heller, O. von Helversen, F. Mayer, R. Neems, P. Rogers, N. Tregenza, and N. Wedell. We are grateful to NERC for funding; to M. Ritchie and J. Mallet for stimulating discussions; and to them, D. Schluter, and an anonymous reviewer for comments on the manuscript.

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